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Structure and function of the selenium translation element in the 3′-untranslated region of human cellular glutathione peroxidase mRNA

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ABSTRACT

In eukaryotes, incorporation of selenocysteine into the polypeptide chain at a UGA codon requires a unique sequence motif, or "selenium translation element" (STE), located in the 3′-untranslated region of the mRNA. The present study examines structure–function relationships of conserved sequence elements and of the putative stem-loop secondary structure in the STE of human *GPX1* mRNA, which encodes the important antioxidant enzyme cellular glutathione peroxidase (EC 1.11.1.9). Deletion of the basal stem, upper stem, or apical loop of the stem-loop structure eliminated the ability of the STE to direct selenocysteine incorporation at the UGA codon of an epitope-tagged *GPX1* reporter construct transfected into COS1 cells. However, mutations that change the primary nucleotide sequence of nonconserved portions of the stem-loop, but preserve its overall secondary structure, by inversion of apical loop sequences or exchange of 5′ and 3′ sides of stem segments, had little or no effect on selenocysteine incorporation. Effects of single- and double-nucleotide substitutions in three short, highly conserved elements in the *GPX1* STE depended in large part on their computer-predicted perturbation of the stem-loop and its midstem bulge. Only in the conserved "AAA" apical loop sequence did mutations show major effects on function without predicted changes in secondary structure. Our results demonstrate the critical role of the three short, highly conserved sequences. However, outside of these elements, the function of the human *GPX1* STE appears to depend strongly on the stem-loop secondary structure.

Keywords: mRNA secondary structure; selenium; translation; translational regulation

INTRODUCTION

Selenoproteins of both prokaryotic and eukaryotic origin are characterized by the presence of the unusual amino acid selenocysteine. Most are enzymes, such as bacterial formate dehydrogenases (Zinoni et al., 1986, 1990), the mammalian glutathione peroxidase (GSH-Px) family (Chambers et al., 1986; Takahashi & Cohen, 1986; Mullenbach et al., 1987; Esworthy et al., 1991), and mammalian type I iodothyronine 5′-deiodinase (Berry et al., 1991b), all of which contain a selenocysteine moiety within the active site. Mammalian selenoprotein P, the major carrier of selenium in serum, contains 10 selenocysteine moieties (Read et al., 1990). In the mRNA of these selenoproteins, the codon UGA, formerly known as one of the three translation termination signals, is used as an exclusive codon for this rare amino acid (Burk & Hill, 1993; Cohen & Avissar, 1993).

In eukaryotes, incorporation of selenocysteine into the polypeptide chain at a UGA codon requires a unique sequence element, termed the selenium translation element (STE), located in the 3′-untranslated region (3′-UTR) of the mRNA (Berry et al., 1991a, 1991b). Most selenoprotein transcripts contain one selenocysteine codon and one STE, except for selenoprotein P, which encompasses 10 UGA codons in the coding region and 2 STE's in the 3′-UTR (Hill et al., 1991; Burk & Hill, 1994). Computer-generated secondary structure analysis of this region in the human type I iodothyronine deiodinase transcript has indicated the probable formation of a stem-loop structure, with at least one

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"bubble" of unpaired bases in the stem (Berry et al., 1991b). Comparison of the sequences of STE's in rat and human iodothyronine deiodinase, GSH-Px, and selenoprotein P mRNAs (Berry et al., 1991a, 1993; Berry & Larsen, 1993) has revealed similar overall secondary structures, but conservation of only three very short sequences: AAA (initially identified as UAAA, located in or near the apical loop); UG (initially identified as UGAU), at or near a bubble on the 3′ side of the stem; and AUGA, at or near a bubble on the 5′ side of the stem (Fig. 1).

The purpose of the present study is to understand the roles of specific sequence and of secondary structure in the stem, apical loop, and three conserved sequences in the function of the STE. We have examined these structure–function relationships in the STE of human *GPX1* mRNA, which encodes the important antioxidant enzyme cellular glutathione peroxidase (Flohe, 1985; Mullenbach et al., 1987; Sukenaga et al., 1987; Shen et al., 1993).

RESULTS

We have previously demonstrated that deletion of either the UAAA or UGAU conserved sequences from an epitope-tagged human *GPX1* cDNA construct resulted in a total loss of the selenium translation element's ability to direct selenocysteine incorporation at the coding region UGA codon (Shen et al., 1993), and have since found that deletion of the AUGA sequence element also abrogated selenocysteine incorporation (data not shown). In addition, other preliminary experiments showed normal expression of an epitope-tagged *GPX1* construct from which all of the 3′-UTR except the 87-nt stem-loop region had been deleted (data not shown). These findings confirmed that the stem-loop region of the 3′-UTR is necessary and sufficient for selenocysteine incorporation and that the three conserved sequences are critical to its function.

To examine the contribution of major sections of the stem-loop structure to selenocysteine insertion into GSH-Px, we tested three epitope-tagged human *GPX1* cDNA constructs with deletions of the basal stem, the upper stem, and the nonconserved apical loop sequence of the structure (Fig. 1). Figure 2 presents an autoradiograph of GSH-Px immunoprecipitated from transfected COS-1 cells. The first lane shows a single band representing endogenous GSH-Px in COS-1 cells transfected with the vector pCMV4 alone. The second lane shows the slightly larger, epitope-tagged *GPX1* gene product in cells transfected with a construct containing the wild-type *GPX1* STE. However, this slower-migrating band was not detectable in lanes 3, 4, or 5, representing cells transfected with the three partial deletion constructs. Thus, each major structural feature of the stem-loop, specifically the basal and upper segments of the stem and apical loop, are essential for the function of *GPX1* STE.

We next tested whether STE function could be maintained in constructs that contained major changes in

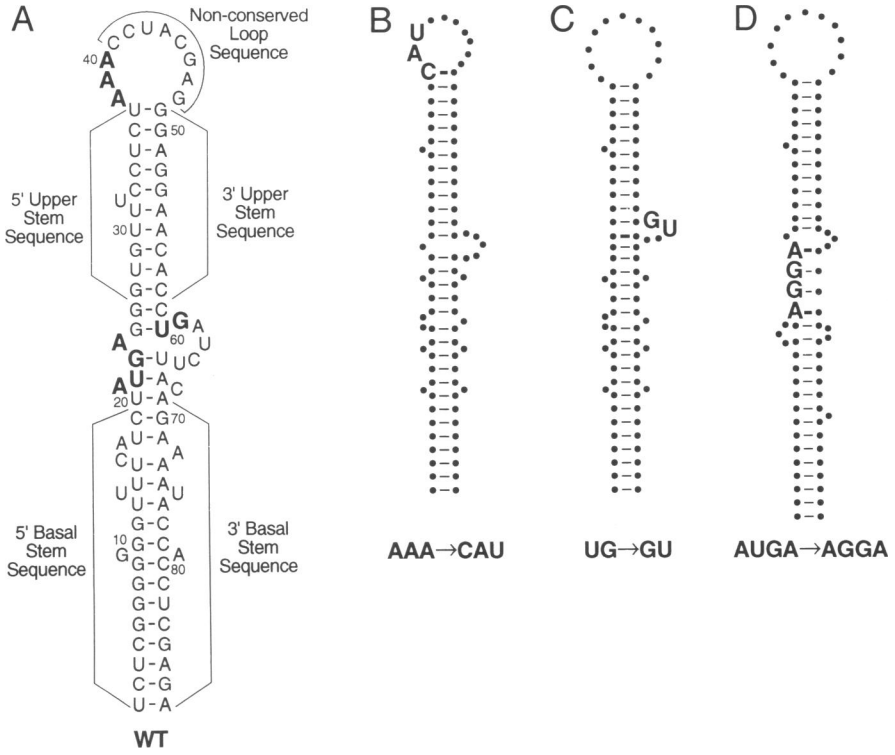

FIGURE 1. Computer (FOLDRNA program) predictions of stem-loop secondary structures of normal and mutated STE's of *GPX1* mRNA. **A:** Predicted wild-type stem-loop structure, with designation of sites of conserved sequences, stem exchanges, and apical loop inversion. **B:** Predicted stem-loop structure for conserved sequence mutation AAA → CAU. Base pairing of the first C of CAU with nucleotide 49-G adds a base pair in the upper stem and reduces the size of the apical loop. **C:** Predicted stem-loop structure for conserved sequence mutation UG → GU. The substitution causes the loss of a base pair between the nucleotide 25G and the substituted 60U, plus two new base-pairings: 25-G with 64-C and 24-A with 65-U, with resultant disruption of the midstem "bubbles." **D:** Predicted stem-loop structure for conserved sequence mutation AUGA → AGGA. Nucleotide numbering in this figure starts with the "U" at the 5′ end of the stem-loop, corresponding to nucleotide 935 of the cDNA sequence (Mullenbach et al., 1987).

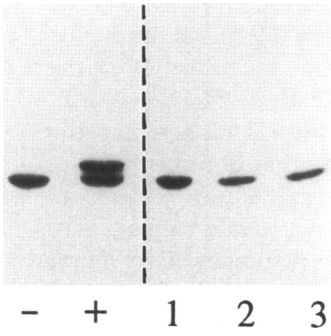

FIGURE 2. Effects of deletions of the basal stem, upper stem, and the nonconserved loop sequences on the function of the *GPX1* STE. Autoradiograph of a representative SDS-PAGE of immunoprecipitated endogenous glutathione peroxidase (lower bands) and transfected, epitope-tagged glutathione peroxidase (upper bands). Lane −, mock-transfected COS-1, negative control; lane +, wild-type *GPX1* STE, positive control; lane 1, basal stem deletion; lane 2, upper stem deletion; lane 3, nonconserved apical loop deletion.

the primary nucleotide sequence of nonconserved portions of the stem-loop but preserved its overall secondary structure. For that purpose, two epitope-tagged *GPX1* cDNA constructs with mutations in the STE were made: in one, the right and left arms of the upper stem were exchanged; in the other, the nonconserved loop sequence of the STE was inverted (Fig. 1). The FOLDRNA program of the Genetics Computer Group software (Devereux et al., 1984) predicted that these mutations would not perturb the overall secondary structure. As shown in Figure 3, when the stem exchange construct was expressed in COS-1 cells, epitope-tagged *GPX1* expression reached levels comparable with the wild-type control construct. The apical loop inversion construct also directed selenocysteine incorporation into GSH-Px (Fig. 3), but quantitative measurements of the level of expression indicated that

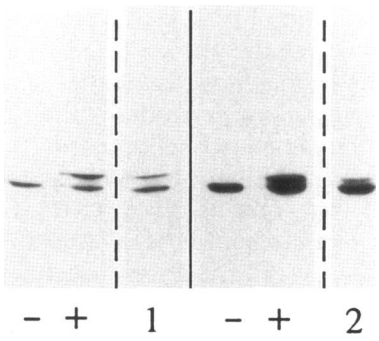

FIGURE 3. Effects of stem exchange and loop inversion on the function of the *GPX1* STE. Autoradiograph of representative SDS-PAGE's of immunoprecipitated endogenous glutathione peroxidase. Lanes −, mock-transfected COS-1, negative control; lanes +, wild-type *GPX1* STE, positive control; lane 1, upper stem exchange; lane 2, nonconserved apical loop sequence inversion.

the level of expression was 56–72% of that provided by the wild-type STE. Thus, the overall secondary structure of these segments appears sufficient to permit translation of the coding region UGA as selenocysteine, but some specific sequence or steric information in the nonconserved portion of the apical loop may also be important for STE function.

We further examined the role of specific nucleotide sequence in the three short, highly conserved elements in the *GPX1* STE. Single- and double-nucleotide substitutions were performed in these conserved sequences and examined for their functional effects on the transient expression of epitope-tagged *GPX1* in COS-1 cells. In parallel, we computer analyzed the possible secondary structure perturbations by each substitution. The results are summarized in Table 1 and described below.

For the apical loop region conserved sequence, AAA, most of the single-nucleotide substitutions, the single-nucleotide deletion, and one of the double-nucleotide substitution (AGG) resulted in varying degrees of partial loss of selenocysteine incorporation activity. However, the other two double-nucleotide substitutions caused a total loss of the function. One point mutation (AGA) preserved normal function, indicating some tolerance for substitution even within this highly conserved sequence.

A similarly wide range of effects was found with nucleotide substitutions in the second conserved sequence, UG. The single-base mutations resulted in a loss of about 50% of selenocysteine insertion function and the double substitutions resulted in a total loss of *GPX1* translation. Thus, STE function in the *GPX1* gene can tolerate most single-nucleotide mutations in these two conserved sequences, but is greatly diminished by additional substitutions.

The function of *GPX1* STE was more sensitive to nucleotide substitutions in the third conserved sequence, AUGA. As shown in Table 1, five single substitutions for each of the first three nucleotides, as well as two double substitutions in this region, all resulted in a total loss of STE function. In the last conserved nucleotide of the short sequence, substitutions of G and U for the wild-type A had no major effect on function, but the substitution of C for the A totally abolished selenocysteine incorporation.

These results confirm the previous deletion experiments in the demonstration of the importance of the three short conserved nucleotide sequences for STE function. However, the substitution mutations also indicate differences in the apparent stringency of the requirements for each sequence element. Reading of the 5′ stem-conserved sequence AUGA appeared to be the most stringent, with no tolerance for substitutions in the first three bases. The requirement for the apical loop-conserved sequence AAA was less stringent, because a single-nucleotide deletion and all single and

TABLE 1. Effects of mutations in the *GPX1* STE on selenocysteine incorporation into epitope-tagged glutathione peroxidase.[a]

Mutation	Relative *GPX1* expression[b] (%)	Predicted perturbation of secondary structure[c]
Upper stem deletion[d]	0,0	Yes
Basal stem deletion[e]	0,0	Yes
Nonconserved loop deletion[f]	0,0	Yes
Upper stem exchange[g]	100,106	No
Nonconserved loop inversion[h]	72,56	No
AAA → GAA	38,30	No
AAA → ACA	35,89	No
AAA → AGA	98,92	No
AAA → AUA	39,77	No
AAA → GAA	38,30	No
AAA → AA_	72,61	No
AAA → AGG	57,33	No
AAA → CAU	0,0	Yes
AAA → GAU	0,0	No
UG → CG	54,60	No
UG → UA	40,44	No
UG → AA	0,0	Yes
UG → GU	0,0	Yes
AUGA → GUGA	0,0	Yes
AUGA → AAGA	0,0	Yes
AUGA → AGGA	0,0	Yes
AUGA → AUCA	0,0	Yes
AUGA → AUGC	0,0	Yes
AUGA → AUGG	108,112	No
AUGA → AUGU	77,59	No
AUGA → GGGA	0,0	Yes
AUGA → UCGA	0,0	Yes

[a] Nucleotide numbering in this table starts with the "U" at the 5′ end of the stem loop, corresponding to nucleotide 935 of the cDNA sequence (Mullenbach et al., 1987). All results represent duplicate independent experiments.

[b] Levels of selenocysteine incorporation relative to simultaneous control transfection of epitope-tagged *GPX1* with the wild-type 3′-UTR.

[c] Computer analysis using the FOLDRNA program of the Genetics Computer Group, Inc., software package.

[d] Deletion of nt 26–37 and 49–59, as indicated in Figure 1A.

[e] Deletion of nt 1–20 and 69–87, as indicated in Figure 1A.

[f] Deletion of nt 41–48, as indicated in Figure 1A.

[g] Complementary exchange of 3′ and 5′ sides (nt 26–37 and 49–59, respectively) of the upper stem, as indicated in Figure 1A.

[h] Inversion of nt 41–48, as indicated in Figure 1A.

even one double substitution were tolerated to some degree.

To evaluate the effects of the small sequence changes on secondary structure of the STE, we examined the predicted structure of the 87-nt segment by FOLDRNA program analysis of each mutation (Table 1; Fig. 1).

For mutations of the AAA sequence segment, computer analysis predicted that only one of the mutations, the double substitution CAU, resulted in a local secondary structure perturbation. As shown in Figure 1, this construct allowed formation of an additional base pair on the top of the upper stem, between the first C of CAU and nucleotide 49-G at the 3′ end of the nonconserved loop sequence (nucleotide numbering in this figure starts with the "U" at the 5′ end of the stem loop, corresponding to nucleotide 935 of the cDNA sequence (Mullenbach et al., 1987)).

For the conserved UG sequence, the FOLDRNA program predicted that both of the double substitutions that inhibit STE function, but not the innocuous single substitutions, would cause a local perturbation of the secondary structure at the midstem bulge. As shown in Figure 1, the double substitutions caused a loss of a base pair between the nucleotide 25-G and the substituted 60-U, plus two new base pairings between 25-G and 64-C and between 24-A and 65-U. Nucleotide 24-A is part of the conserved sequence AUGA that is normally unpaired in the wild-type *GPX1* STE.

For mutations within the third conserved sequence, computer analysis further predicted that all the detrimental single and double substitutions for the first three nucleotides A, U, and G, plus the single substitution of C for the last nucleotide A, resulted in local secondary structure perturbations (Table 1; Fig. 1), whereas the innocuous single substitutions of G or U for the last nucleotide A did not alter local secondary structure.

These results indicate a strong correlation between the functional effects of nucleotide substitutions within the three short conserved sequences of the *GPX1* STE and their effects on the secondary structure of the stem-loop. Mutations that perturbed the secondary structure of the stem-loop and its midstem bulge profoundly affected STE function, but sequence changes that preserved secondary structure had little or no effect on selenocysteine incorporation. The only exceptions to the latter rule were mutations in the conserved apical loop sequence, AAA (e.g., AAA → GAA, AAA → GAU).

DISCUSSION

We have examined the relationships of primary nucleotide sequence and of predicted secondary structure with the function of the human *GPX1* STE, which directs the translational incorporation of selenocysteine into cellular glutathione peroxidase (Shen et al., 1993). The STE's of mammalian selenoprotein genes share a common overall stem-loop structure, containing three very short, 2–4-nt consensus sequences (Fig. 1). Our results indicate that function of the human *GPX1* STE depends on the overall secondary structure, including the basal stem, the upper stem, and apical loop components. However, the primary nucleotide sequences of these parts proved less critical. Our results also confirm the critical role of the three conserved sequences, as previously indicated by our prior studies of *GPX1* (Shen et al., 1993) and by the detailed mutagenesis of the type I 5′-deiodinase gene performed by Berry and

Larsen (1993). The current analysis of these regions also suggests a correlation between STE function and the local secondary structures surrounding these three conserved sequences: AAA, UG, and AUGA; Fig. 1). That is, the functional effects of mutations in these elements depended largely, but not exclusively, on whether or not the substitutions produced changes in the predicted base pairing pattern of the stem-loop.

Major changes in STE sequence that preserve secondary structure, such as exchange of stem sequences or inversion of the nonconserved portion of the loop, have little effect or only partially diminish STE function. In contrast, single-nucleotide mutations that disrupt the predicted secondary structure can abolish selenocysteine incorporation into glutathione peroxidase protein. Berry and Larsen (1993) have also reported dependence on secondary structure, rather than specific sequence, in the distal section of the upper stem of the rat 5′ deiodinase STE.

Even in the short conserved sequence elements UG and AUGA, the structure–function experiments suggest that *GPX1* STE function is sensitive to changes in secondary structure, and tolerant of mutations that change the sequence but preserve the local base pairing patterns. However, mutations in the sequence element AAA, at the beginning of the apical loop region, markedly diminished or abolished STE function, even if they did not change the predicted secondary structure. This finding implies the presence of specificity for sequence, not just secondary structure, in this section of the stem-loop. The remainder of the apical loop may also contain some less stringent sequence requirements, because inversion of the entire nonconserved portion only partially preserves STE function.

These conclusions, based on secondary structures predicted by the program FOLDRNA, must be considered with several caveats. First, of course, the program makes predictions based on free energy of base pairing, and so the structures represent models, not representations of experimentally verified configurations. Furthermore, recognition by putative RNA-binding proteins and the operation of the STE probably depend upon still more complex tertiary structure, which is not considered by the program.

A comparison to published computer-predicted secondary structures reveals differences between the STE's of eukaryotic selenoprotein mRNAs both within and between species (Berry et al., 1991a; Berry & Larsen, 1993). For example, in human *GPX1* and the rat 5′-deiodinase STE, the conserved AAA is located in the loop region; but in the rat *GPX1* STE, the first A forms a base pair in the upper stem. Similar diversity also takes place in the fine structure of the bulges around the UG and AUGA sequences in human *GPX1* (Fig. 1) and rat 5′-deiodinase (Berry & Larsen, 1993) STE's. These differences could indicate variation between species or even tissues in the specificities of STE recognition elements or in the formation of secondary or tertiary structures in differing intracellular environments. Such variation could provide a means for eukaryotes to achieve optimal translational regulation of individual selenoprotein genes. The elucidation of these differences will ultimately depend on the identification of the specific eukaryotic protein factor(s) involved in STE recognition and function.

The conservation of the three sequence elements, despite the tolerance for some substitutions shown in the present study, implies either some specificity for primary sequence information at those locations or a need for these elements in the formation of more complex tertiary structures such as loop folding (Marino et al., 1995) or pairing between loops.

The mechanism by which the STE directs translation of the coding region UGA remains unknown. In prokaryotes, genes encoding selenoproteins, such as *E. coli* formate dehydrogenase, contain a critical stem-loop structure immediately downstream from the UGA codon (Zinoni et al., 1990). In *E. coli*, a selenocysteine-specific translation elongation factor, SELB, has been identified and found to form a tertiary complex with the stem-loop element and the charged selenocysteyl-tRNA (Forchhammer et al., 1989). This elongation factor presumably interacts with the ribosomal translational machinery, resulting in the selenocysteine incorporation into the polypeptide chain of formate dehydrogenase. By analogy, it is likely that mammalian RNA-binding proteins also interact with the STE, as well as the selenocysteyl-tRNA and ribosomal assembly, in order to suppress termination and allow selenocysteine translation at appropriate UGA codons in selenoprotein gene transcripts. Our data suggest that such putative protein(s) recognize both secondary structure and specific sequence information in the STE of human *GPX1* transcripts.

MATERIALS AND METHODS

Construction of epitope-tagged GPX1 cDNA subclones

Unless otherwise indicated, *GPX1* deletion subclones were constructed by overlap extension PCR (Ho et al., 1989), using a Perkin-Elmer Cetus thermal cycler and reagents. This PCR method required two flanking primers defining the size of the final product and two mutually complementary primers directing the desired mutation in the target sequence. The final PCR products were inserted back into pBluescript KS, the sequences confirmed, and then the mutant *GPX1* sequence inserted into the eukaryotic expression vector pCMV4 (Andersson et al., 1989) for transfection into COS-1 cells as described below. For construction of subclones with deletion of the STE basal stem, two consecutive runs were conducted to complete the deletion. For construction of subclones with single- and double-nucleotide substitutions in the small conserved sequences, the mutually complementary oligo-

nucleotide primers directing a particular substitution were synthesized with a mixture of deoxy-A, C, G, and T at the desired substitution position(s), and mutagenesis performed either by PCR as above or using the Altered Sites II in vitro Mutagenesis System (Promega).

The products of the oligonucleotide-directed mutagenesis were inserted back into pBluescript KS carrying the epitope-tagged *GPX1* cDNA clone via *Avr* II and *Xba* I sites. After reconfirmation of the sequence, the subclones were transferred to the eukaryotic expression vector pCMV4 via the *Cla* I and *Xba* I sites.

Transfection, labeling, and lysis of COS-1 cells

COS-1 cells were transfected for transient expression of GSH-Px subclones by modified calcium phosphate-mediated or electroporation methods (Maniatis et al., 1990), then cultured in DMEM medium supplemented with 10% fetal bovine serum, 5 ng/mL sodium selenite, 25 mM HEPES, pH 7.4, and 1× penicillin–streptomycin-fungizone (Gibco-BRL). All experiments were performed 2–4 times.

In preliminary experiments to test transfection efficiency, COS-1 cells were cotransfected with 2 μg of plasmid pXGH5 included in a human growth hormone transient expression assay system supplied by Nichols Institute. Human growth hormone secreted into the medium was detected by radioimmunoassay using the Crystal Multidetector RIA System (United Technologies Packard). Within each experiment, calcium phosphate transfection efficiency was sufficiently similar that subsequent correction for efficiency between constructs was not necessary.

"Epitope tagging" of GSH-Px was performed as described previously (Shen et al., 1993) by replacing the first 12 nt of the open reading frame of *GPX1* with a 30-nt sequence encoding an ATG start codon followed by 27 bases encoding a nine-amino acid epitope of human influenza hemagglutinin protein (Kolodziej & Young, 1991). Although rabbit antiserum against this epitope was available, its binding to tagged GSH-Px molecule was much lower than that of the antisera against GSH-Px peptide sequences (data not shown), so the latter was still used to detect the tagged GSH-Px molecule.

Immunoprecipitation and protein electrophoresis

Immunoprecipitation utilized two rabbit antisera raised (by Berkeley Antibody Co., Richmond, California) against synthetic peptide sequences from the GSH-Px polypeptide chain, one from residues 26 to 46 and the other from residues 174 to 192. Fifteen microliters of each antiserum plus 20 μL of protein A-Sepharose CL-4B beads (Sigma) were added to the lysate. The mixture was processed as described previously (Shen et al., 1993) and the immunoprecipitate collected for SDS-PAGE. Protein electrophoresis on 12% SDS-polyacrylamide gels and autoradiography were performed by standard techniques (Maniatis et al., 1990). Band densitometry of ^{75}Se-autoradiographs on Amersham Hyperfilm-Mp was analyzed by ImageMeasure software (Microscience, Inc.), as described previously (Newburger et al., 1994).

For each STE mutant construct, at least two independent transfections were conducted and analyzed.

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